THE REGULATION OF ASPARTOKINASE DURING GROWTH AND SPORULATION OF *BACILLUS CEREUS*

An abstract of a Thesis by
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The problem. Diaminopimelic acid (DAP) and dipicolinic acid (DPA) are thought to be important chemical constituents in the formation of heat-resistant spores in baccilli. They are formed through a multistep biosynthetic pathway in which aspartokinase is the first enzyme. The aspartokinase activity in *Bacillus cereus* has been shown to be repressed by lysine and yet still be able to form heat-resistant spores with near normal amounts of DPA. This study was undertaken to attempt to elucidate how the products formed through the functioning of aspartokinase in *B. cereus* could be present when the enzyme had been repressed to low levels of lysine.

Procedure. Cells of *B. cereus* T were grown by batch method in lysine assay medium (LAM) with or without lysine added. The cells were harvested and broken by sonication or a French press and the cell-free extracts assayed for aspartokinase activity in various buffers at different times along the growth curve. For inhibition studies, the inhibitors were added to the assay mixture.

Findings. A high ionic strength buffer containing 4M NaCl that was tested revealed the presence of aspartokinase activity that was not repressed or inhibited by lysine but was sensitive to feedback inhibition by DAP.

Conclusions. *B. Cereus* is found to have two isozymes of aspartokinase. Aspartokinase I (AK I) is repressed and inhibited by lysine. It is the predominant enzyme functioning during vegetative growth of *B. cereus* cells. AK II is sensitive to feedback inhibition by diaminopimelate but insensitive to repression or inhibition by lysine. AK II functions during sporulation to ensure channeling of carbon compounds to DPA and DAP for sporogenesis. AK II is rapidly inactivated in most standard buffers; however, a 4M NaCl buffer was developed to stabilize the enzyme for assay.

THE REGULATION OF ASPARTOKINASE DURING GROWTH AND SPORULATION OF *BACILLUS CEREUS*

A Thesis

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Carl David Smith

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INTRODUCTION AND REVIEW OF LITERATURE

Vegetative cells of the genus Bacillus form a heat resistant and metabolically inert spore when the supply of certain nutrients can no longer support growth. The process of sporulation in response to external stimuli offers a relatively simple model for studying the mechanism of cellular regulation of differentiation. It is hoped that elucidation of these mechanisms may provide insight into understanding cellular control of development, morphogenesis, and neoplasia in eucaryotic systems.

Stages of Sporulation

Young and Fitz-James (1959) have described seven morphological events that occur during sporulation in Bacillus cereus. These events are similar for other species of Bacillus (Schaeffer, 1969).

Stage I. The chromatin bodies of the vegetative cell coalesce to form a single axial rod.

Stage II. The cytoplasmic membrane invaginates to divide the cell unequally into two compartments. In addition, the septation by the double layered membrane divides the genetic material.

Stage III. As the double membrane elongates, it surrounds the smaller compartment to form a forespore polarly displaced in the sporangium or mother cell. The germ cell wall is synthesized on the external side of the inner

membrane.

Stage IV. Synthesis of cortex peptidoglycan occurs between the double layered membrane and external to the germ cell wall.

Stage V. Two layers of protein surround the cortex and are known as the spore coat. It is believed to protect the spore from physical injury and enzymatic degradation.

Stage VI. The spore acquires heat resistant properties and accumulates dipicolinic acid and calcium.

Stage VII. The sporangium lyses and the mature spore is released.

Control of Sporulation

Control of sporulation by catabolite repression was first suggested by Schaeffer et al. (1965). According to this hypothesis, utilization of carbon and nitrogen containing metabolites through catabolic pathways exhausts the compound and results in derepression of genes coding for sporulation specific enzymes. In continuous cultures of B. subtilis grown on minimal media, sporulation is initiated after glucose or nitrogen depletion limit growth; however, sporulation does not occur when citrate, tryptophan, phosphate, or Mg^{++} depletion limit growth (Dawes and Mandelstam, 1970).

Elmerich and Aubert (1975) have proposed repression of sporulation in B. megaterium by a low molecular weight effector synthesized through a biosynthetic pathway in

which glutamine synthetase, converting glutamate to glutamine, is the first enzyme. Mutants of B. megaterium containing defective glutamine synthetase were unable to sporulate (Reysset and Aubert, 1975). Twelve mutants were isolated that required glutamine for growth; five of these mutants were able to utilize glutamate instead of glutamine. Reysset and Aubert propose that glutamine synthetase binds with the low molecular weight effector formed through its functioning and represses sporulation. Mutants, therefore, could be defective in their catalytic properties and be unable to form the low molecular weight effector or have a glutamine synthetase that will not bind the effector. In B. cereus and B. licheniformis, AMP strongly inhibits glutamine synthetase (Hubbard and Stadtman, 1967). Conceivably, as the energy source of growing bacilli is depleted, the intracellular levels of AMP rise to shut down the catalytic activity of glutamine synthetase and prevent synthesis of the effector. Such a system would have its advantages to the metabolism of the cell since purine and pyrimidine synthesis would be prevented and energy and carbon would be conserved in the form of glutamate. The derepression of glutamate oxaloacetate and glutamate pyruvate transaminases in early sporulation require glutamate for the formation of amino acids for the spore coat (Buono et al., 1966).

In Escherichia coli the lac operon is regulated by

catabolite repression through glucose which prevents the formation of cyclic AMP. When glucose has been exhausted, the cyclic AMP levels within the cell rise and act on the promotor site of the operon to facilitate transcription (Pastan and Perlman, 1970). Clark and Bernlohr (1972) have detected cyclic AMP in sporulating cells but not the vegetative cells in B. licheniformis. Setlow (1973), however, was not able to detect cyclic AMP, adenyl cyclase, or cyclic AMP phosphodiesterase in B. cereus T, B. megaterium, B. licheniformis, or B. subtilis SB 1933 in either vegetative or sporulating cells. These results support the work by Ide (1971) using B. subtilis IFO 3025 and B. cereus 3027.

The finding by Keilman et al. (1976) that netropsin, an inhibitor of DNA and RNA polymerase, blocks sporulation of B. subtilis without preventing derepression of three catabolite repressed enzymes suggests that the de novo formation of sporulation specific enzymes may be ancillary in the control of sporulation.

Losick and Sonenshein (1969) reported that RNA polymerase from sporulating cells of B. subtilis was not able to transcribe phage ϕe DNA but RNA polymerase from the vegetative cells would transcribe B. subtilis phage ϕe DNA. A change in template specificity was found to occur on the RNA polymerase within the first hour of sporulation (Losick et al., 1970). It was found that the 57,000 dalton sigma subunit was lost from the core polymerase in the sporulating

cells. The sigma subunit is formed during sporulation but binds poorly to the core RNA polymerase (Tjian and Losick, 1974). Binding of the sigma subunit to the core can be restored by the addition of chloramphenicol, an inhibitor of protein synthesis, to sporulating cells (Segall et al., 1974). A sporulation protein with protease activity has been proposed to inhibit sigma binding to the core polymerase during sporulation (Segall et al., 1974). RNA polymerase from vegetative and sporulating cells of B. cereus T. displays no change in template specificity for transcribing B. cereus phage ϕCL, and a 56,000 dalton sigma subunit is found to bind tightly to both enzymes (Rexer et al., 1975).

Importance of the Aspartic Acid Family Pathway

Dipicolinic acid is unique to the spores of bacteria. It may comprise as much as 15% of the dry weight of the spore and is found in a 1:1 ratio with Ca^{++} (Murrell, 1969). It has long been thought that chelation of Ca^{++} by dipicolinic acid (DPA) in the cortex of the spore confers heat-resistant properties to the spore. Mutants of B. cereus T lacking DPA, however, have been shown to form heat-resistant spores (Hanson et al., 1972). Thermoresistance of these mutants was lost after storage in distilled water and they were unable to germinate efficiently in liquid media. Fitz-James (1971) has shown that Ca^{++} ions stabilize the

phospholipids of the spore membrane since the core is nearly anhydrous. When the core is rehydrated during germination, Ca^{++} must be chelated from the membrane for the successful formation of vegetative cells. Dipicolinic acid is thought to be responsible for this function.

Diaminopimelic acid (DAP) is found in the vegetative cells of most species of Bacillus and is present in the cortex of the spore in all Bacillus species. It cross-links the L-Ala-D-Glu-m-DAP-D-Ala residues of the N-acetyl muramic acid of the glycan chains (Schleifer and Kandler, 1972). Warth et al. (1963) have shown a correlation between DAP content of spores and heat resistance. This suggests that the degree of cross linking may be responsible for thermoresistance of the spore.

Both meso-diaminopimelic acid and dipicolinic acid are synthesized through the biosynthetic pathway of the aspartic acid family amino acids in B. cereus T. (Aronson et al., 1967). The pathway is shown in Fig. 1. The operation and control of this pathway is critical during sporulation to ensure the synthesis of DAP and DPA for the spore. In B. cereus T, aspartokinase, aspartic beta-semialdehyde, dihydrodipicolinic synthase, and dihydrodipicolinate reductase all increase in specific activity during sporulation (Forman and Aronson, 1972; Hoganson and Stahly, 1975a, 1975b). Diaminopimelate decarboxylase activity is repressed by the intracellular lysine pool during exponential growth;

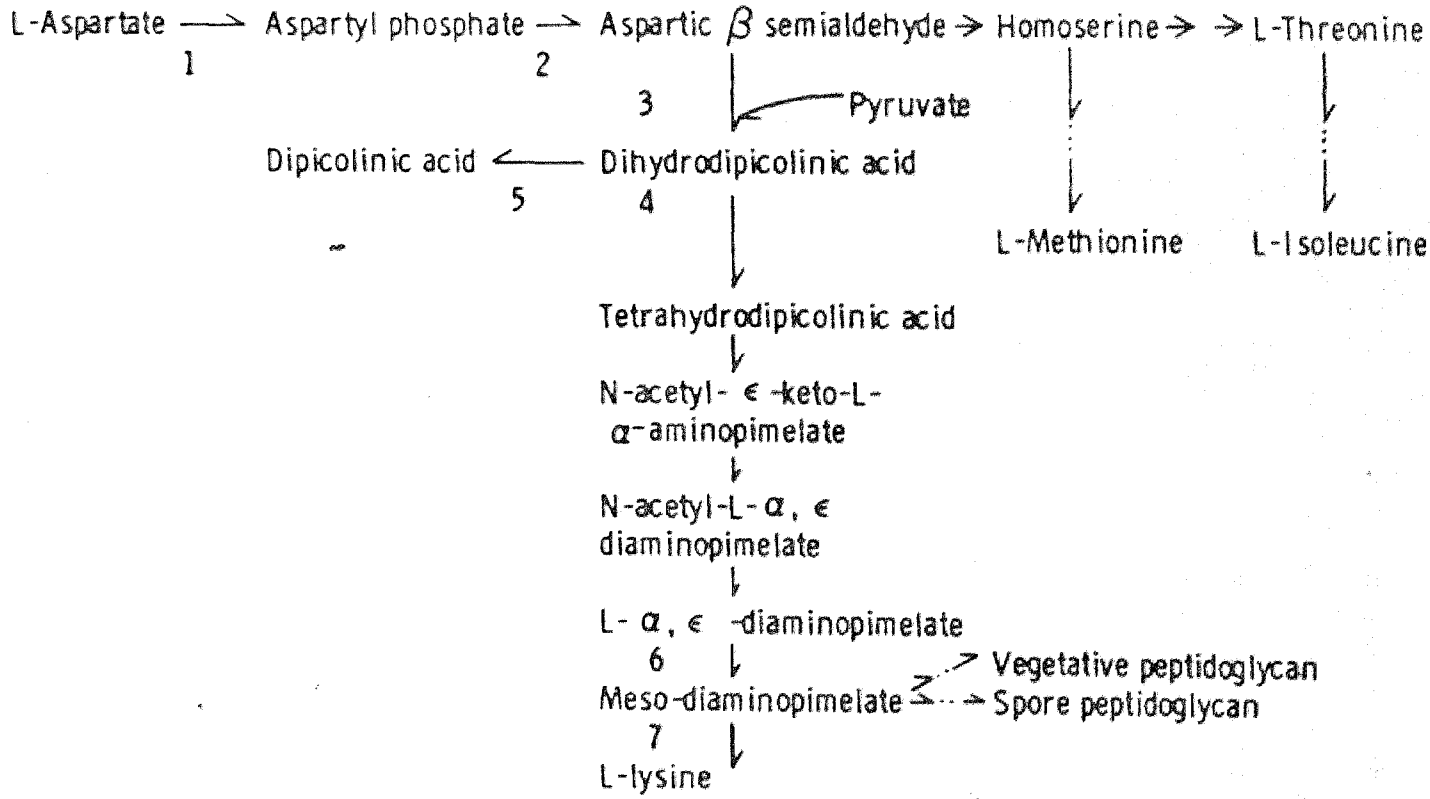

Fig. 1. 1. Aspartokinase; 2. Aspartic β semialdehyde dehydrogenase; 3. Dihydrodipicolinic acid synthase; 4. Dihydrodipicolinic reductase; 5. Dipicolinic acid synthase; 6. Diaminopimelic acid epimerase; 7. Diaminopimelic acid decarboxylase.

however, by the time DPA synthesis begins during the late stages of sporulation, diaminopimelate decarboxylase activity is completely repressed regardless of the size of the intracellular lysine pool (Grandgenette and Stahly, 1971). Dipicolinic acid synthase has only been detected during sporulation (Bach and Gilvarg, 1966).

The result of repression of some enzymes and derepression of others during sporulation is the channeling of carbon atoms into diaminopimelic acid and dipicolinic acid. A threefold rise in the intracellular level of DAP occurs in B. cereus T. prior to the synthesis of DPA (Grandgenette and Stahly, 1971). Dihydrodipicolinic acid (DHDPA) is the branch point at which carbon atoms can go to DPA or DAP. In late sporulation, a high percentage of ^{14}C-aspartate is channeled into dipicolinic acid; however, diaminopimelate synthesis persists (Rogers et al., 1972). The high rate of synthesis of dipicolinate was postulated to be due to an increase of synthesis of DHDPA rather than the shut down of the alternate pathway. This was supported by Forman and Aronson (1972) who found DHDPA reductase, the first enzyme leading from DHDPA to DAP, to be insensitive to feedback inhibition by DAP or DPA. Studies on DHDPA synthase by Hoganson and Stahly (1975a) failed to show regulation or modulation of that enzyme by lysine or combinations of amino acids.

Control of Aspartokinase in Bacteria

As can be seen in Fig. 1, aspartokinase is the first enzyme in the biosynthetic pathway to L-lysine, L-methionine, L-threonine, L-isoleucine, diaminopimelic acid, and dipicolinic acid. In many multi-step biochemical pathways, the first enzyme limits the rate of formation of the end product. It is thought that such control may exist for the aspartate family biosynthetic pathways. In Escherichia coli, three aspartokinases are found (Cassan et al., 1975). Aspartokinase I is repressed and inhibited by lysine (Stadtman et al., 1961). Aspartokinase II is repressed by methionine (Patte et al., 1967) and aspartokinase III is inhibited by threonine and repressed by threonine and isoleucine in concert. However, E. coli does not form spores and cannot synthesize DPA. The functioning of the pathway to lysine, DPA, and DAP is not as critical in stationary phase cells of non-sporulating bacteria as it is in Bacillus species.

Cells of B. polymyxa are found to contain an aspartokinase that is subject to multivalent inhibition by lysine plus threonine (Paulus and Gray, 1967). Multivalent inhibition by end products in concert indicates that the inhibition by the effectors together is greater than the sum of the inhibition of each effector alone. In the case of B. polymyxa, threonine or lysine alone do not inhibit the enzyme. Repression of the enzyme was seen when the

cells were grown in rich media; however, lysine, threonine, and methionine did not repress aspartokinase activity in minimal media (Biswas et al., 1970). Comparisons of enzyme from log and sporulating cells of B. polymyxa have not been reported. The aspartokinase of B. colistinus is also multivalently inhibited by lysine plus threonine (Ito et al., 1969). A single aspartokinase in B. licheniformis was reported by Stahly and Bernlohr (1967) to be inhibited by L-lysine, by L-aspartic beta-semialdehyde, and by L-lysine plus L-threonine in concert. When these cells are grown in the presence of lysine, aspartokinase loses its sensitivity to feedback inhibition by L-lysine (Gray and Bernlohr, 1969). The change in sensitivity to lysine inhibition was thought to be due to alterations of the enzyme rather than the production of a second enzyme.

In B. brevis, B. stearothermophilus, and two strains of B. subtilis evidence exists for the presence of two separate aspartokinases. Hitchcock and Hodgson (1976), working with B. brevis, have shown the two aspartokinases to be similar in their Michaelis-Menton kinetics and stabilization properties. One is repressed and inhibited by lysine and the other is repressed by threonine and inhibited by lysine plus threonine in concert. Uninhibitable aspartokinase activity was thought to be due to alteration of the multivalently inhibited enzyme rather than a third aspartokinase. Work by Kuramitsu and Yoshimura

(1972) indicates that B. stearothermophilus contains one aspartokinase that is inhibited by lysine in concert with threonine and another enzyme that is inhibited by meso-DAP. The meso-DAP sensitive aspartokinase is derepressed about the same time DPA synthesis begins. Similarly, Hampton et al. (1971) have reported one aspartokinase that is inhibited and repressed by lysine in B. subtilis 60015 which declines in activity during sporulation and a second enzyme which maintains its activity throughout sporulation. The latter aspartokinase is not inhibited by a variety of amino acids including lysine. Lysine plus threonine acting in concert inhibit the vegetative aspartokinase in B. subtilis ATCC 6051. This strain also produces a sporulation specific aspartokinase which is inhibited by meso-DAP.

The aspartokinase of B. cereus T. when grown on a media devoid of lysine has a high specific activity during log growth, drops precipitously as the cells enter stationary phase, and then increases in specific activity during late sporulation (Hoganson and Stahly, 1975b). The biosynthesis of DPA has been localized in the mother-cell compartment of sporulating B. cereus (Andreoli et al., 1975). Because forespores were not observed to be involved with DPA biosynthesis, aspartokinase activity is also probably located in the mother cell compartment. Forman and Aronson (1972) and Aronson et al. (1967) found a single aspartokinase in cells of B. cereus T. The enzyme from

vegetative cells was inhibited by lysine alone. However, aspartokinase from extracts of sporulating cells was not inhibited by lysine. An alteration of the vegetative enzyme was proposed to account for the insensitivity to inhibition by lysine of the late enzyme. A second enzyme was ruled out since a mutant with a reversion rate of a point mutation was auxotrophic for lysine, had a low aspartokinase activity, and was deficient in the DPA content of its spores. However, DAP levels of the mutant were normal, and DPA added to the medium did not significantly increase the DPA content of the spores. Hoganson and Stahly (1975b) reported that the aspartokinase of B. cereus T is inhibited by lysine during both growth and sporulation, and this is in direct conflict with the findings of Forman and Aronson (1972) that aspartokinase from sporulating cells of B. cereus T was insensitive to inhibition by lysine.

The aspartokinase of B. cereus T was found to be repressed to very low levels in all stages of growth and sporulation when the media contained 10 mM L-lysine (Hoganson and Stahly, 1975b). Experiments were performed to determine if ^{14}C-aspartate would be converted to DPA in cells in which aspartokinase had been repressed by lysine. Aspartate was found to be incorporated at nearly the same rate as the control, and the spores formed were found to retain their heat resistant properties. No explanation was offered to explain how carbon atoms from ^{14}C-aspartate

could be channeled to DPA at normal levels when aspartokinase had been repressed to low levels by lysine in the culture media.

Purpose of the Research

The functioning of the biosynthetic pathway of the aspartic acid family amino acids is required for the formation of amino compounds important in the structure and physiology of the spore. This research reexamined the control of aspartokinase, the first enzyme of this pathway, in B. cereus T. The finding of Hoganson and Stahly (1975b) are in direct conflict with those of Aronson et al. (1967) and Forman and Aronson (1972) with respect to control of this enzyme. One study shows normal amounts of final product formed when the first enzyme of the pathway leading to this product, aspartokinase, is thought to be nearly absent (Hoganson and Stahly, 1975a). Also this study hoped to elucidate methods of stabilizing the enzyme from inactivation because aspartokinase of B. cereus T is very unstable and differences in the findings of the investigators may be due to an unstable aspartokinase.

METHODS AND MATERIALS

Bacillus cereus strain T was used to study the regulation of aspartokinase. The organism was obtained from Dr. D. P. Stahly at the University of Iowa.

Growth and Sporulation Media

Modified G-Media. G-medium, developed by Greenberg (1954) was modified by doubling the potassium phosphate and glucose content (Hoganson, 1974). The composition of the modified G-medium is given below.

Compound	Percent (w/v)
$MnSO_4 \cdot H_2O$	.005
$FeSO_4 \cdot 7H_2O$	.00005
$CuSO_4 \cdot 5H_2O$	.0005
$ZnSO_4 \cdot 7H_2O$	.0005
$MgSO_4$	.02
$(NH_4)_2SO_4$	.20
$CaCl_2 \cdot 2H_2O$	.008
K_2HPO_4	.10
Glucose	.20
Yeast Extract	.20

A stock mineral solution was prepared by combining 40% $(NH_4)_2SO_4$ to an equal volume of a solution containing 1.0% $MnSO_4 \cdot H_2O$, 0.01% $FeSO_4 \cdot 7H_2O$, 0.1% $ZnSO_4 \cdot 7H_2O$ and 4.0% $MgSO_4$. Separate stock solutions of 0.8% $CaCl_2 \cdot 2H_2O$, 5.0% K_2HPO_4, 10% glucose and 10% yeast extract were prepared and autoclaved at 121 C for 15 minutes.

The modified G-medium was prepared by autoclaving a mixture of mineral stock solution, the amount equal to 1.0% of the final volume of the media, with distilled water equal to 92% of the final media volume. After letting the solution cool, sterile stock solutions of glucose, potassium phosphate, yeast extract, and calcium chloride were

added aseptically in amounts equal to 2.0%, 2.0%, 2.0% and 1.0% of the final volume, respectively. Growth of B. cereus in this medium is limited by one of the components within the yeast extract.

Lysine Assay Media. Lysine assay media (LAM) was prepared for the growth of B. cereus by adding five milliliters of an inorganic salt solution to be described below and 8.5 ml of 20% (w/v) KH_2PO_4 to 7.8 g of LAM. The pH of this mixture was adjusted to 7.2 with concentrated KOH, and the media was brought to a final volume of 500 ml with distilled water. The media was autoclaved at 121 C for 10 minutes rather than 15 minutes to reduce decomposition of media components. The stock solution of inorganic salts has been described by Vinter (1963) and is prepared as follows:

Compound	g/l
K_2SO_4	17.4
$MgSO_4 \cdot 7H_2O$	12.3
$MnSO_4 \cdot 2H_2O$	0.134
$FeSO_4 \cdot 7H_2O$	2.0
$ZnSO_4 \cdot 7H_2O$	1.44
$CaCl_2 \cdot 2H_2O$	12.3

When the solution had been thoroughly mixed and brought to its final volume with distilled water, it was filtered once to remove any precipitate that had formed.

Lysine assay media is a rich defined medium which is devoid of lysine. The total lack of lysine in the media

was confirmed by the absence of growth of a mutant of B. cereus auxotrophic for lysine. The addition of lysine to the media allowed growth of the mutant.

Growth and Culture Conditions

Preparation of Spore Stocks. Spore stocks of B. cereus T were prepared by inoculating modified G-medium with spores obtained from Dr. D. P. Stahly. The spores were incubated at 30 C on a shaker for approximately 72 hours to allow for growth, sporulation, and the release of mature spores. At the end of incubation, the spores were centrifuged in a Lourdes Betafuge at 14,000 RPM for 15 minutes. The supernatant was decanted and the spores were washed once in sterile distilled water and recentrifuged. The washed spores were then resuspended in a volume of sterile distilled water equal to 10% of the media volume the cells were grown in, and frozen at -20 C. Spore stocks contained approximately 10^9 spores per ml.

Cultivation of B. cereus T for the Aspartokinase Assay. Cells of B. cereus T that were to be assayed for aspartokinase activity were grown in LAM prepared as above. The LAM was inoculated with one ml of a 10^{-6} dilution of spore stock and incubated at 30 C in a New Brunswick Scientific Co. rotary shaker. When the cells reached an A_{600} of 0.3 to 0.5 or a turbidity of 80 to 100 Klett units, they were transferred to fresh medium in a volume equal to 14% of the final volume of the new culture. The subculture

was incubated under the same conditions and again a 14% transfer was made to fresh LAM when the absorbance at 600 nm was between 0.3 and 0.5. The procedure was performed once more to insure synchronously growing cells of B. cereus and the cells from this culture were tested for aspartokinase activity.

Other Experimental Techniques

Microscopic examination of the cultures were periodically made with a phase contrast microscope to determine stages of growth and sporulation and the efficacy of cell breakage. The turbidity of the cultures was determined by reading the absorbance at 600 nm with a Bausch and Lomb Spectronic 20, or by using a Klett-Summerson Photoelectric Colorimeter with a #54 green filter (500-575 nm). A Corning Model 10 pH meter (Corning Scientific Instruments, Corning, New York) was employed for measuring pH.

Preparation of cell extracts of Bacillus cereus T. Growing or sporulating cells of B. cereus T were harvested at different times along their growth curve by centrifuging the media at 14,000 RPM at 4 C with a Lourdes Betafuge for 15 minutes. The cells were then washed once in buffer. The washed cells were frozen until broken for the aspartokinase assay. Breakage of cells was initially performed with a Branson Model W185 sonicator (Branson Sonic Power Co., Danbury, Connecticut) at a setting of six for three minutes.

Breakage of the cells was 80-90% effective; however, it was thought that the intense heat generated by the sonicator may be inactivating some of the enzyme despite the ice bath surrounding the cells. For later experiments, a modified French pressure cell (Walter and Stahly, 1968) was used for breaking the cells. The broken cells were centrifuged at 20,000 x g for 15 minutes in either the Lourdes Betafuge or a Sharples Super Centrifuge at 4 C. The supernatant was dialyzed for 6-8 hours at 4 C in the same buffer they were washed in. After dialysis, the cell extracts were used immediately for assaying aspartokinase activity.

Buffer. The buffer found to stabilize aspartokinas activity contained 4.0 M NaCl added to a standard buffer that has been used previously (Aronson et al., 1967). The standard buffer contained 0.02 M tris hydrochloride, 0.005 M $MgCl_2$, and 0.15 M KCl plus 0.01 M 2-mercaptoethanol. Other chemicals added to the standard buffer in attempting to stabilize aspartokinase activity included 1 M sucrose, 0.025 M L-aspartic acid, 2 M NaCl, and phenylmethylsulfonyl-fluoride (PMSF). The PMSF was prepared for addition to the buffer by dissolving 6 g into 1 liter of 95% ethanol. The buffer contained a 5% solution of this mixture as had been described by Greenleaf et al. (1973). The final pH of all the above buffers was adjusted with concentrated KOH to pH 8.0.

Protein Determinations. The protein concentrations

of cell extracts were determined by either the biuret assay as described by Gornal et al. (1949), or the coomassie blue protein binding assay of Bradford (1976).

Biuret reagent was prepared by dissolving 2.9 g of cupric sulfate and 18 g of sodium potassium tartrate in 1.5 liters of distilled water. To this, 900 ml of 10% NaOH was added. This mixture was brought to a final volume of three liters, and the reagent was standardized against known amounts of bovine serum albumin. Because the 2-mercaptoethanol in the buffer interferes with the biuret assay for protein, it was removed prior to running the assay by precipitating one ml of the buffered cell extract with 7% (w/v) trichloroacetic acid. The precipitated protein was allowed to incubate at 4 C for 30 minutes, centrifuged at 3000 RPM for 10 minutes, and then washed with another 5 ml of 7% trichloroacetic acid. The washed pellet was resuspended with one ml of 0.2 N NaOH and then assayed with the biuret reagent. When the biuret reagent and protein solution had incubated at 37 C for 30 minutes, the absorbance at 540 nm was determined with a Beckman Double Beam Spectrophotometer and the values were compared with the standard curve.

The coomassie blue assay for protein was run by adding 0.1 ml of cell extract to five ml of the coomassie blue reagent. The coomassie blue reagent was standardized with known amounts of bovine serum albumin. Mercaptoethanol

does not interfere with the coomassie blue assay; this permits the direct analysis of the cell extract without a precipitation step. The sensitivity of this assay required that a 10 to 20 fold dilution of cell extract be performed for the values to be significant. After a two minute incubation, the absorbance at 595 nm was recorded and compared against the known standards.

Aspartokinase assay. The enzyme assay for aspartokinase has been described by Stadtman et al., (1961) and employs the replacement of the phosphate group of aspartyl phosphate by hydroxamic acid to form L-aspartic beta-hydroxamate which forms a colored product in the presence of $FeCl_3$. The reaction mixture contained: dialyzed enzyme extract; tris-HCl buffer adjusted to pH 8.0 with KOH, 0.1 mmoles; L-aspartic acid, 0.03 mmoles; ATP, 0.01 mmoles; hydroxylamine-KCl, 0.8 mmoles; and $MgCl_2$, 0.0032 mmoles. The final volume of the reaction mixture was one ml. Hydroxylamine-HCl and L-aspartic acid were neutralized to pH 8.0 with concentrated KOH. The ATP and hydroxylamine-KCl mixtures were prepared just prior to running the assay to minimize their degradation. The stock solutions of the other chemicals were prepared in advance. The reaction was begun with the addition of 0.2 ml of enzyme extract and routinely run for 60 minutes at 26 C. The reaction was stopped by the addition of 1.5 ml of a solution containing 10% $FeCl_3$, 0.7 N HCl, and 3.3% trichloroacetic acid. The

precipitated protein was removed by centrifugation and the amount of aspartyl beta-hydroxamate was determined by comparing the A_{540} of the reaction mixture plus stopping solution to a standard curve prepared with known amounts of aspartyl beta-hydroxamate. Each reaction mixture had its own blank which was identical except that it lacked L-aspartic acid.

One unit of enzyme is defined as that amount catalyzing the formation of one nanomole of aspartic beta-hydroxamate per minute. Specific activity is defined as units of enzyme per milligram of protein in the reaction mixture.

Inhibition studies of aspartokinase contained 0.005 M L-lysine and/or 0.005 M D,L-alpha, epsilon diaminopimelic acid in the reaction mixture unless otherwise stated.

Chemicals

The reagents and biochemicals used in this study are listed below by the manufacturer's name.

Baker Chemical Company Phillipsburg, New Jersey	$CaCl_2 \cdot H_2O$
Difco Laboratories Detroit, Michigan	Glucose Lysine Assay Media Sucrose Yeast Extract
Elmer and Amend Chem. Co. New York, New York	$CuSO_4 \cdot 5H_2O$

Fisher Scientific Company Fairlawn, New Jersey	$FeCl_3$ KCl KOH KH_2PO_4 K_2HPO_4 K_2SO_4 $MgCl_2$ $MgSO_4$ $MgSO_4$ $7H_2O$ $MnSO_4$ H_2O NaCl NaOH $(NH_4)_2SO_4$ $ZnSO_4$ $7H_2O$ Sodium potassium tartrate Trichloroacetic acid
Mallinckrodt Chemical Works St. Louis, Missouri	$FeSO_4$ $7H_2O$
Sigma Chemical Company St. Louis, Missouri	L-aspartic acid L-aspartylbeta-hydroxamsyr Adenosine triphosphate Bovine serum albumin Coomassie blue reagent D,L-alpha, epsilon-diaminopimelic acid Hydroxylamine hydrochloride L-lysine 2-mercaptoethanol Tris-hydrochloride

Pure meso-diaminopimelic acid used for inhibition studies of aspartokinase was a gift of Dr. D. P. Stahly at the University of Iowa.

RESULTS

Validity of the Aspartokinase Assay. The aspartokinase assay of Stadtman et al. (1961) was shown to be valid for the aspartokinase of B. cereus T by measuring the amount of product formed as a function of enzyme concentration and

time of reaction. The amount of aspartyl beta-hydroxamic acid formed during the assay was found to increase linearly with increases in either enzyme concentration (Fig. 2) or time the reaction was allowed to incubate (Fig. 3).

Saturation curves of aspartokinase were determined using aspartic acid (Fig. 4) and ATP (Fig. 5) as substrate. The cell-free extracts of exponentially growing cells were used to study saturation phenomena since there had been no indication from previous work of more than one aspartokinase throughout growth and sporulation in B. cereus T. Using the data from Fig. 4, the concentration of aspartic acid in the reaction mixture was increased from 10 mM to 30 mM.

A major difficulty encountered when studying the aspartokinase of B. cereus T is that it is very unstable. Experiments were designed to determine if the addition of various compounds to the 0.02 M tris-HCl buffer would stabilize aspartokinase activity over time (Fig. 6). In addition to the compounds tested, the reaction mixture contained 10 mM mercaptoethanol which has been shown to help stabilize aspartokinase (Forman and Aronson, 1968). Aspartokinase stability was enhanced by 4.0 M NaCl with 88% of its activity remaining 24 hours after the first assay. Aspartic acid, one of the products of the reaction catalyzed by aspartokinase, phenylmethylsulfonyfluoride, a protease inhibitor, 2.0 M NaCl, and 1.0 M Sucrose were not as effective in retarding degradation of aspartokinase activity.

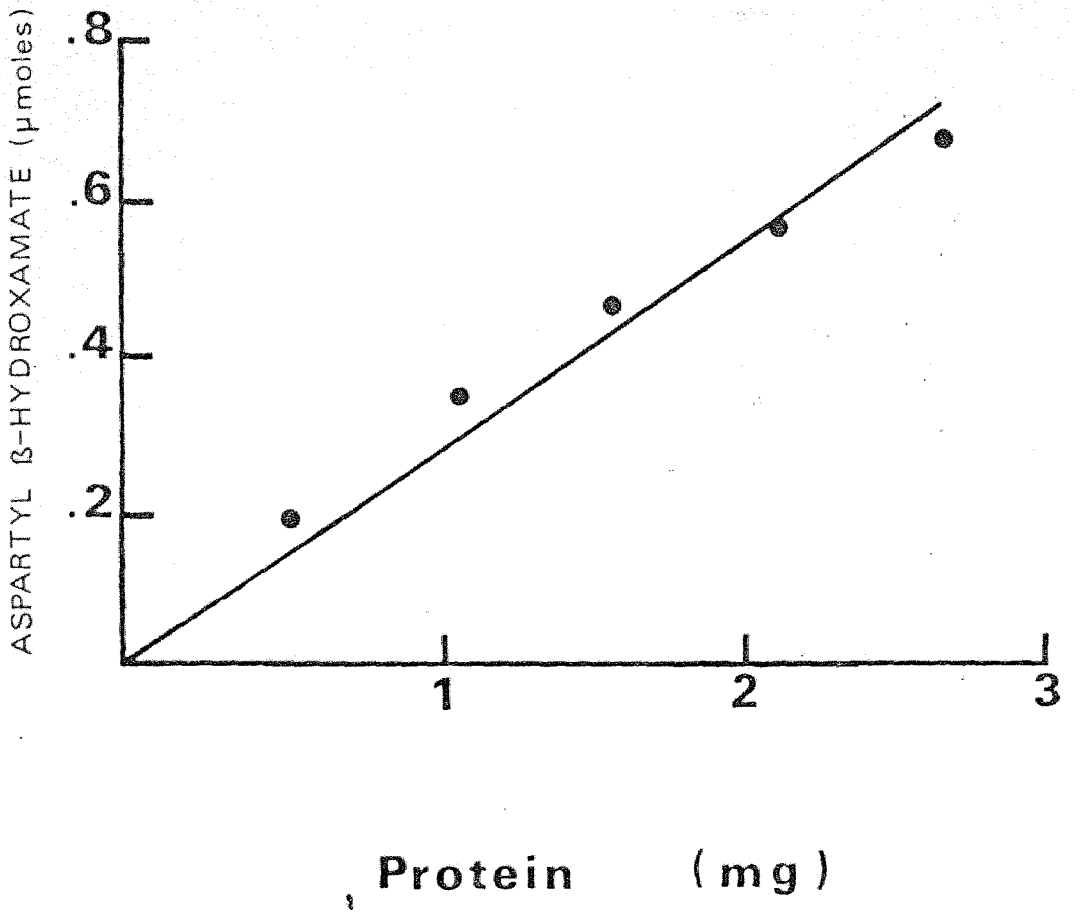

Fig. 2. Aspartokinase activity expressed in umoles of aspartyl B-hydroxamate as a function of enzyme concentration. The cell-free extracts were prepared from cells grown on LAM and harvested during early sporulation. The crude enzyme extract was buffered with 0.02 M tris-HCl with 10 mM mercaptoethanol at pH 7.8. The reaction time of the assay was 60 minutes.

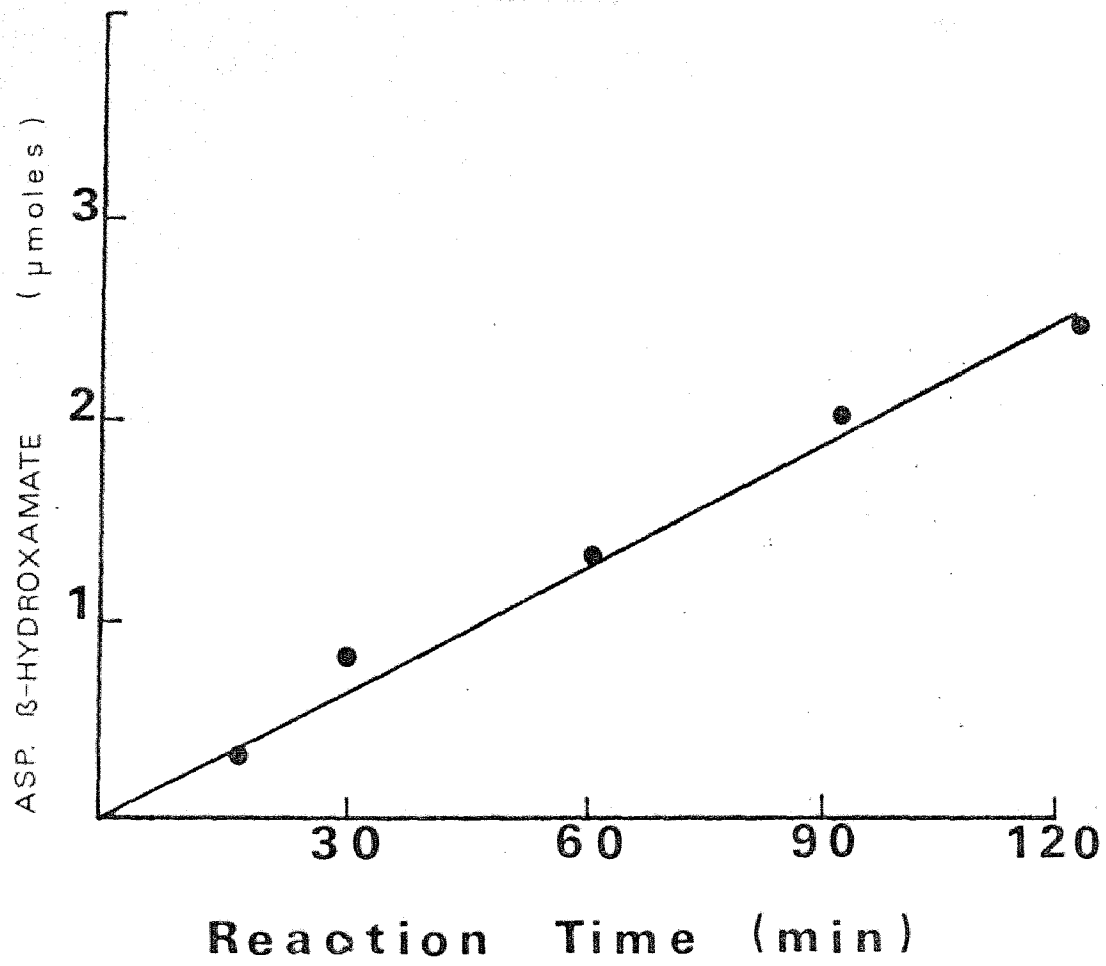

Fig. 3. Aspartokinase activity expressed in umoles of aspartyl B-hydroxamate as a function of reaction time. The cell-free extracts were prepared from cells grown on LAM and harvested at the end of exponential growth. The crude enzyme was buffered with 0.02 M tris-HCl with 10 mM mercaptoethanol at pH 7.8.

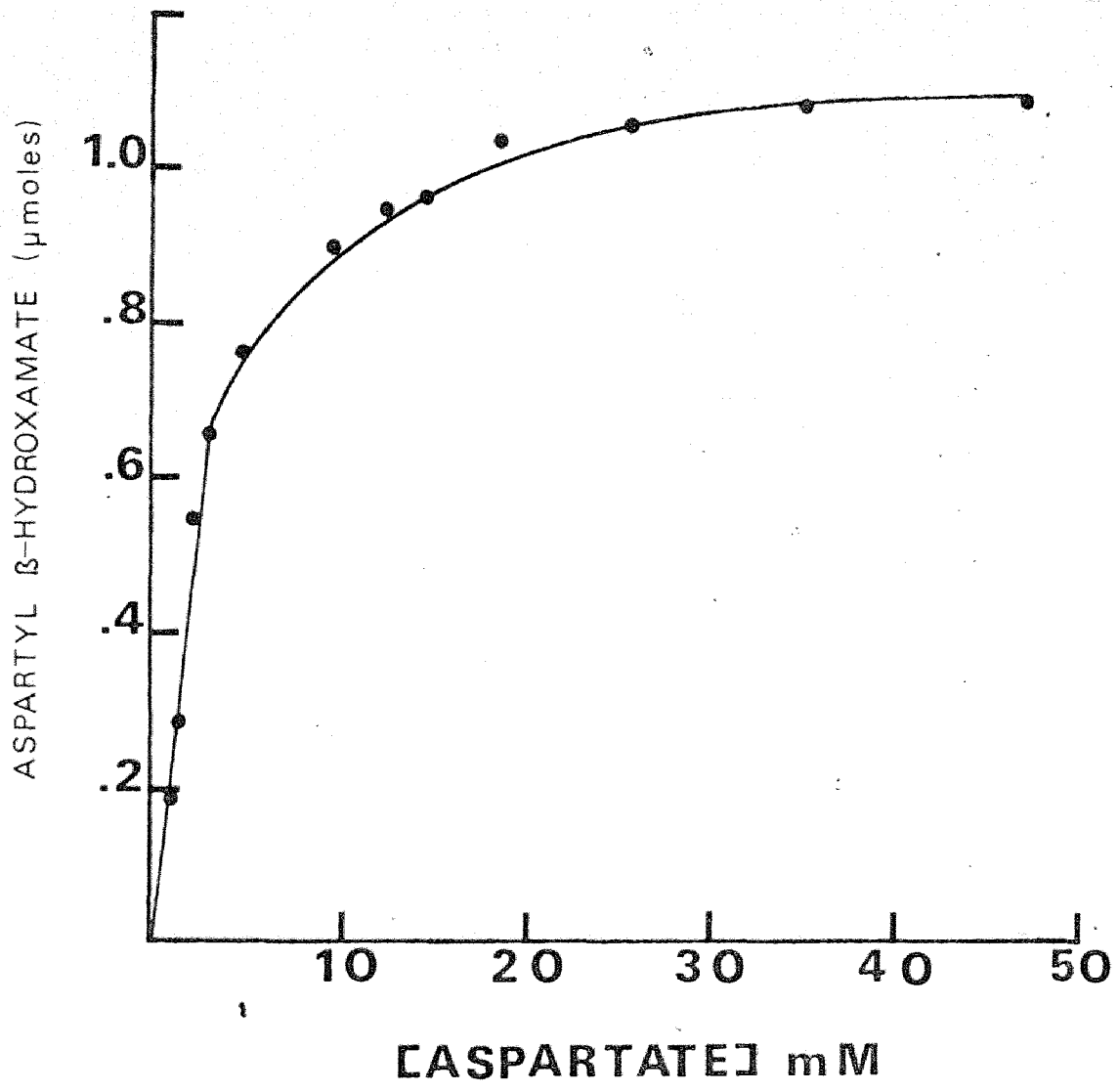

Fig. 4. Aspartokinase activity in umoles of aspartyl B-hydroxamate as a function of aspartic acid concentration. The cells were harvested during exponential growth and broken in 0.02 M tris-HCl buffer plus 10 mM mercaptoethanol. The ATP concentration of the reaction mixture was 14 mM. The reaction was initiated by the addition of 0.2 ml of cell-free extract and the mixture was allowed to incubate at 26 C for 60 minutes.

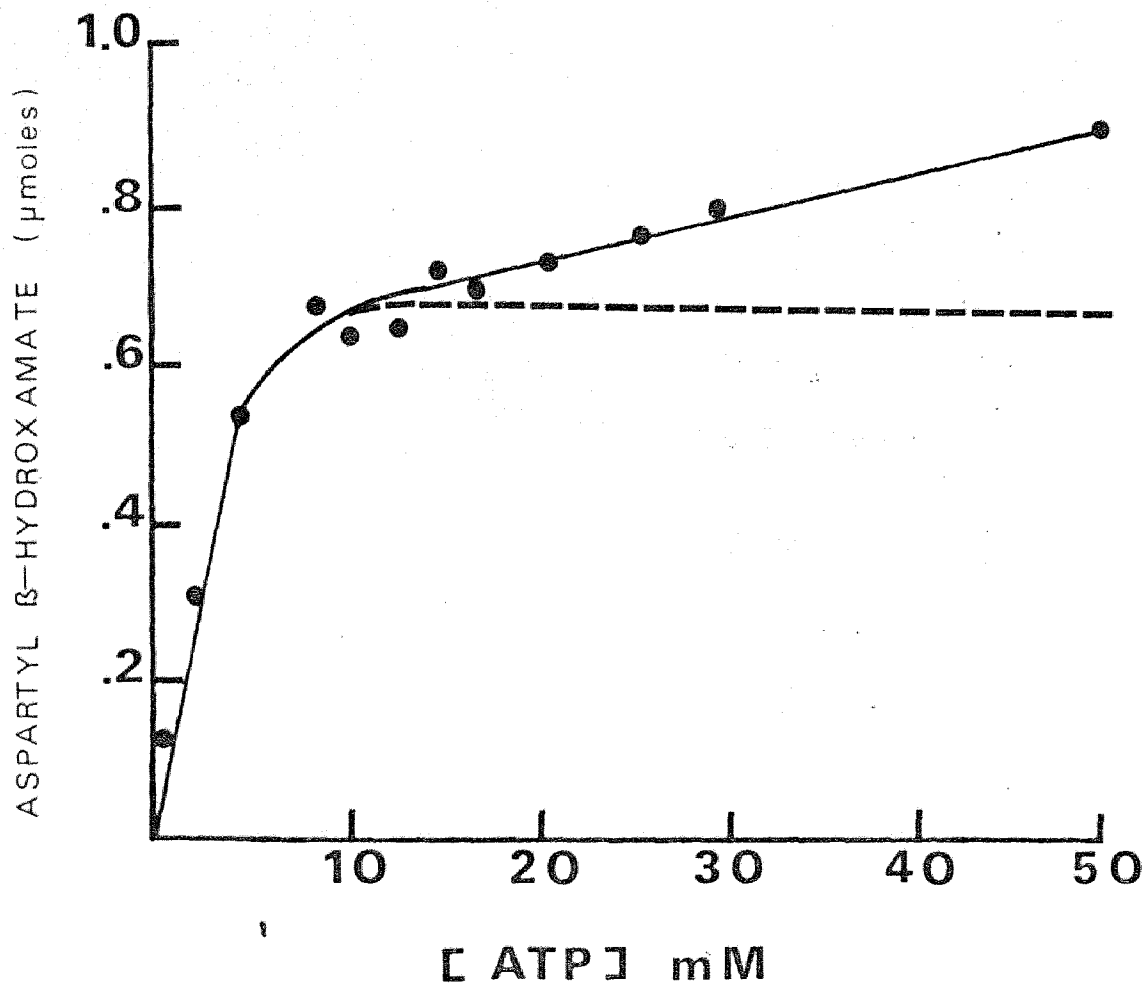

Fig. 5. Aspartokinase activity expressed in umoles of aspartyl B-hydroxamate as a function of ATP concentration. The cells were harvested during mid-exponential growth and broken in 0.02 M tris-HCl plus 10 mM mercaptoethanol at pH 7.8. Aspartic acid concentration of the reaction mixture was 10 mM, however, when it was increased to 50 mM the ATP saturcurve was not significantly altered.

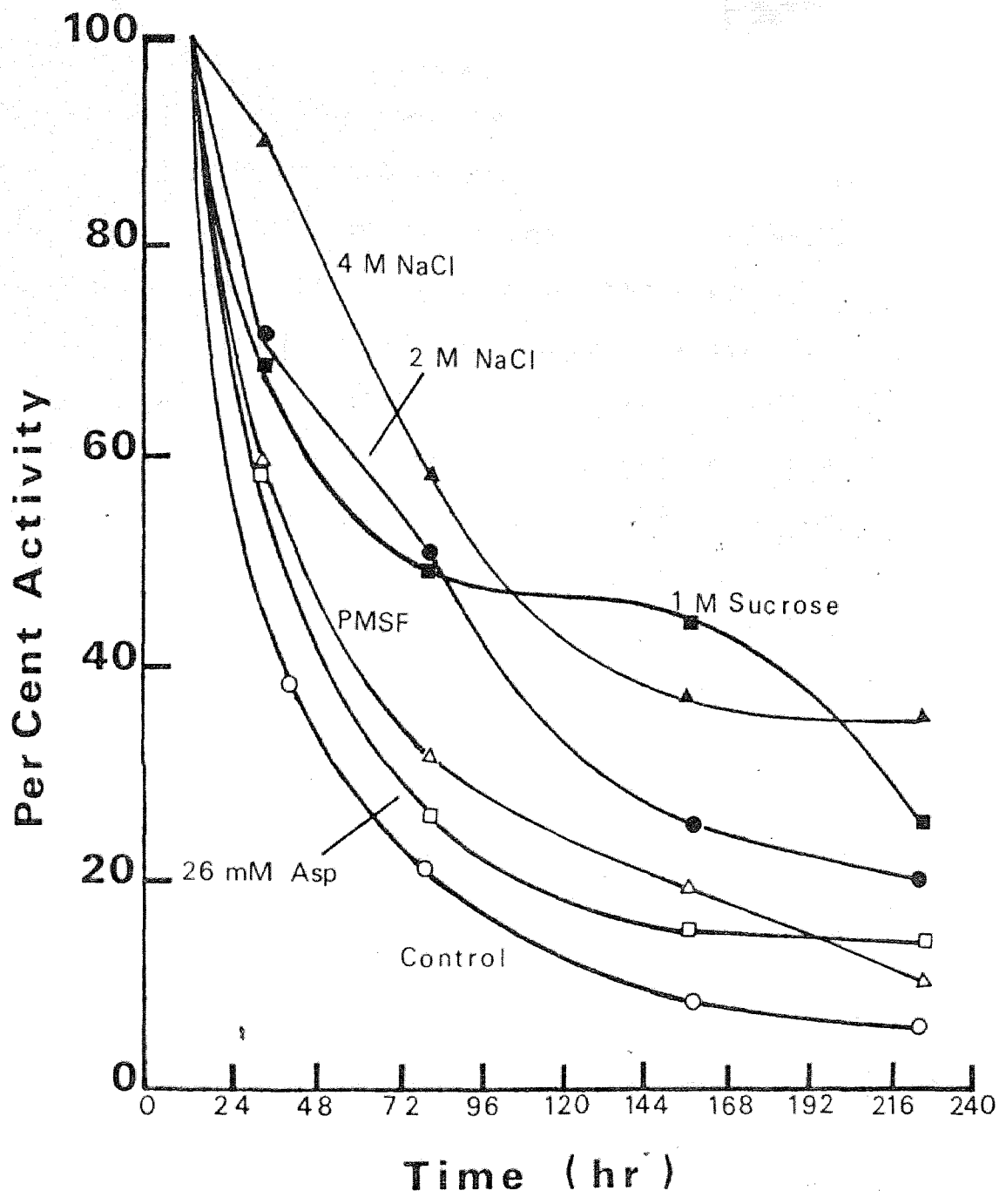

Fig. 6. The effect of phenylmethylsulfonylfluoride (PMSF), 1 M sucrose, 26 mM aspartate, 2 M NaCl, and 4 M NaCl on aspartokinase stability over time. The cells were harvested during exponential growth and broken in 0.02 M tris-HCl with 10 mM mercaptoethanol at pH 7.8. The first assay was performed 8 hours after breakage of the cells and was assigned a value of 100% activity.

The specific activity of aspartokinase during growth and sporulation of B. cereus in the presence and absence of lysine. Figure 7 shows the aspartokinase from B. cereus grown in the absence of lysine had a high specific activity during exponential growth which declined during early stationary phase. During sporulation the specific activity of aspartokinase became derepressed and the specific activity increased. The addition of lysine to the media repressed the specific activity of aspartokinase during log growth and in early stationary phase cells of B. cereus. However, during sporulation, cells grown in lysine overcame repression of aspartokinase activity by lysine and were found to have near normal levels of aspartokinase activity.

Feedback inhibition of aspartokinase from B. cereus during growth and sporulation. The ability of lysine to repress aspartokinase activity from exponential phase cells of B. cereus and not from sporulating cells of the same organism suggested the possibility of more than one aspartokinase. To test this, inhibition studies were undertaken on lysine repressed and lysine free aspartokinase. Fig. 8 shows the effect of these inhibitors on aspartokinase specific activity from cells grown in the absence of lysine. When aspartokinase was assayed in the presence of lysine, the specific activity was inhibited to a low level during exponential growth and throughout stationary phase. However,

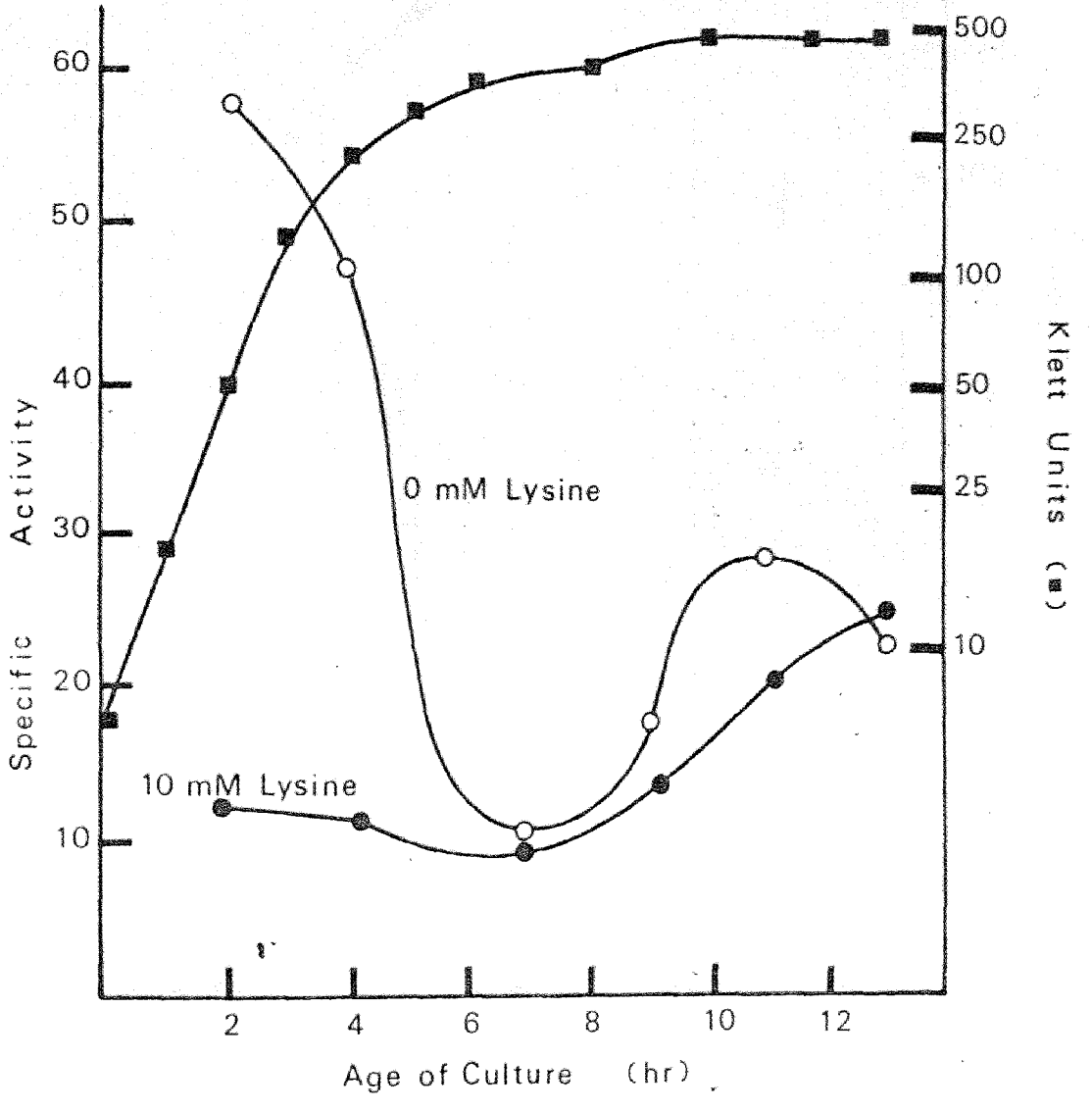

Fig. 7. Specific activity of aspartokinase in B. cereus during various stages of growth and sporulation when grown in LAM (o) or in LAM supplemented with 10 mM L-lysine (o). The cells were broken in 0.02 M tris-HCl buffer at pH 7.8 to which 10 mM mercaptoethanol and 4 M NaCl had been added.

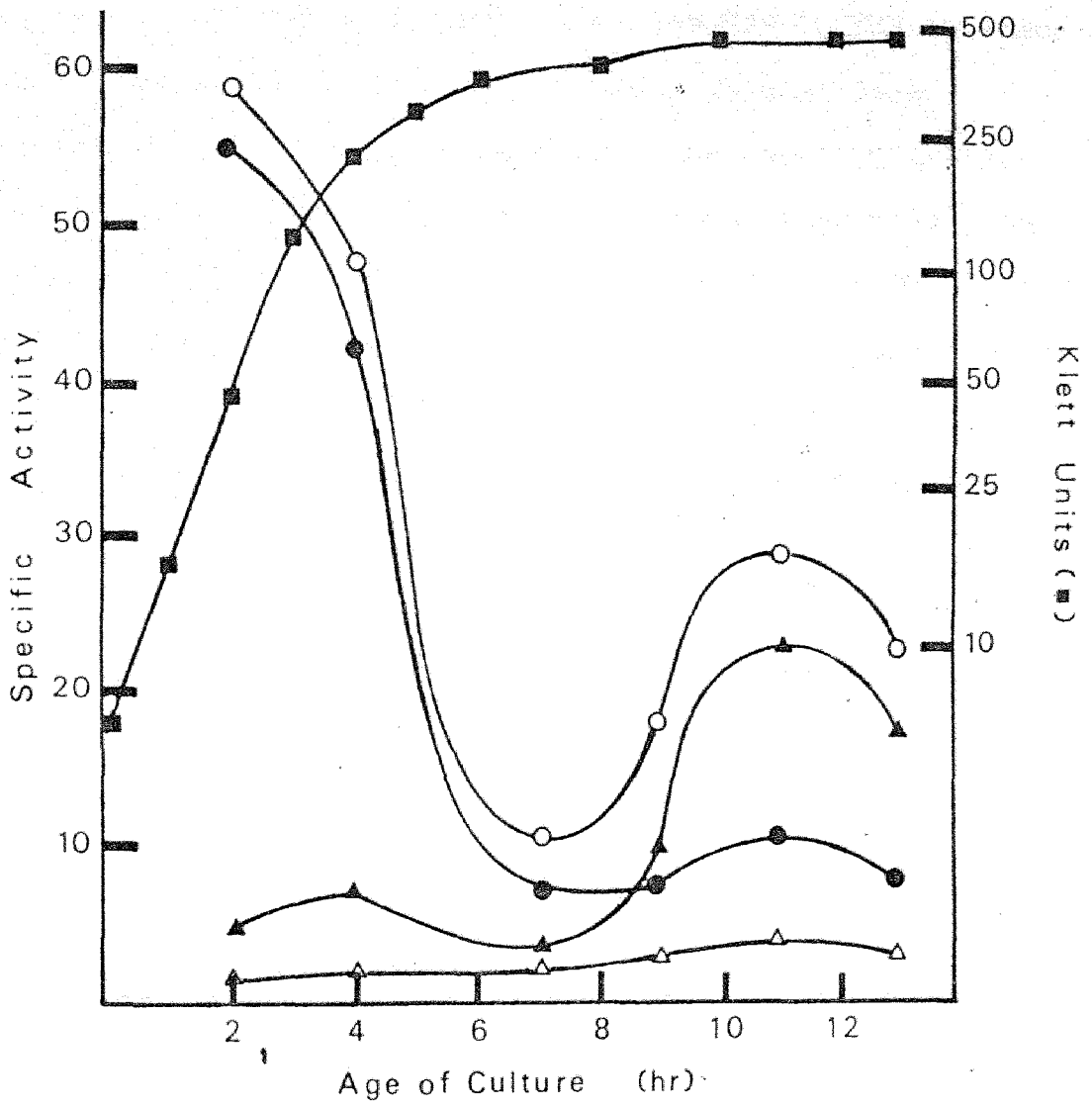

Fig. 8. Inhibition of aspartokinase activity during growth and sporulation of B. cereus when grown on LAM. The uninhibited enzyme used as a control was assayed as described in the Methods and Materials (O). Inhibited enzyme contained 10 mM α,γ meso DAP (●), 10 mM L-lysine (▲), or 10 mM L-lysine plus 10 mM α,ε, meso DAP (Δ) in the reaction mixture. The units of specific activity are in umoles/min/mg of protein.

the specific activity of aspartokinase from sporulating cells of B. cereus grown in the absence of lysine was only slightly inhibited by lysine. When assayed in the presence of DAP, the specific activity of aspartokinase from sporulating cells was inhibited to a low level while the activity from exponential phase cells was essentially that of the uninhibited enzyme. With the addition of DAP plus lysine to the aspartokinase reaction mixture, the specific activity is inhibited throughout growth and sporulation to levels that approximate the sum of inhibition by lysine alone plus inhibition by DAP alone.

Figure 9 shows the effect of inhibitors on aspartokinase specific activity which was not repressed by lysine in the culture media. The effects of lysine repression were most pronounced on enzyme levels during exponential growth, whereas aspartokinase activity increased to near normal levels during the late stages of sporulation. When DAP was added to the assay mixture the enzyme level was inhibited during log growth and most markedly inhibited during sporulation. Lysine inhibited the lysine-unrepressible aspartokinase to about the same degree throughout growth and sporulation of B. cereus. Lysine and DAP together inhibit aspartokinase from B. cereus grown in lysine about as well as the sum of inhibition by lysine alone plus inhibition by DAP alone, but did not completely inhibit

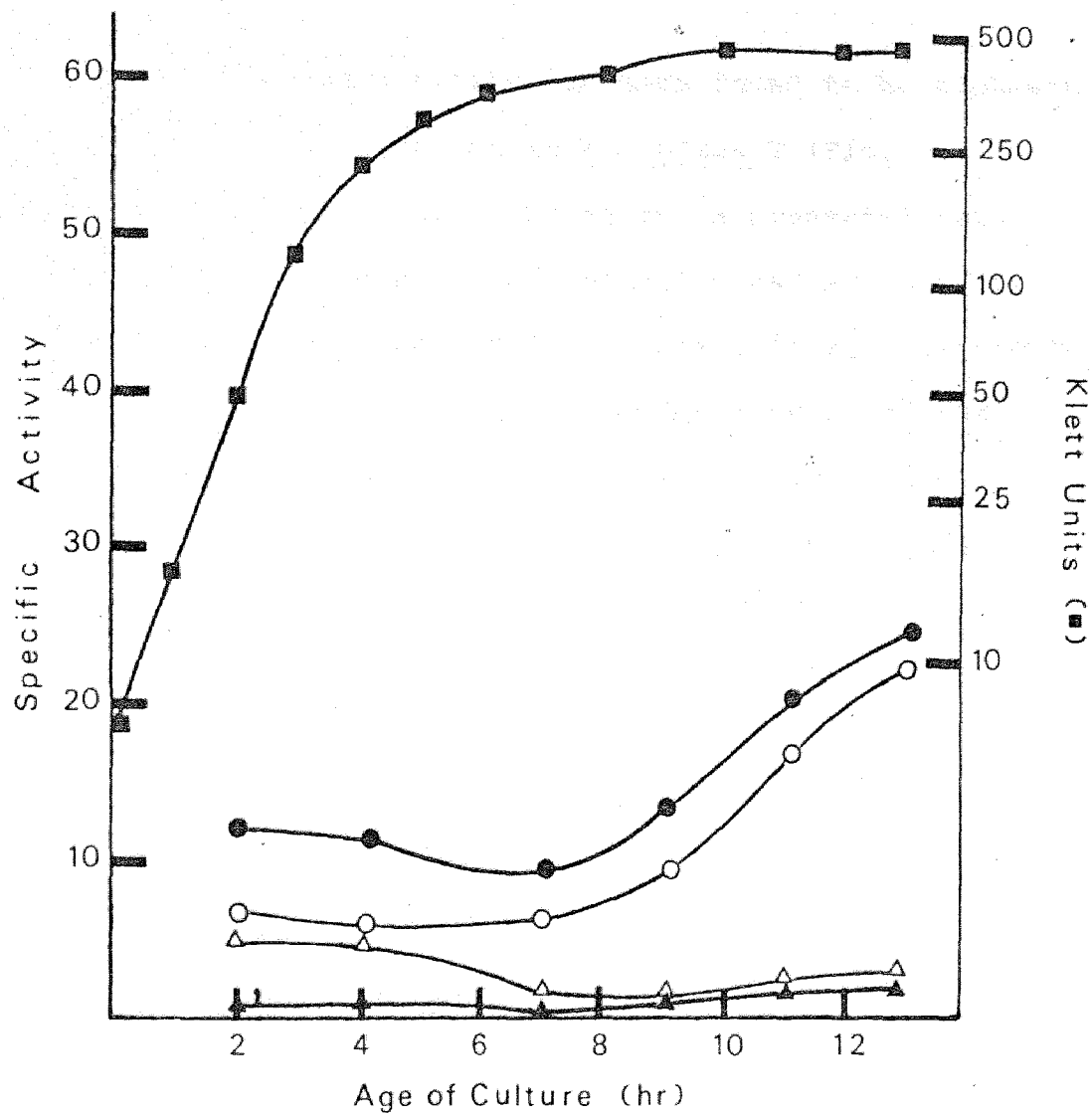

Fig. 9. Inhibition of aspartokinase activity during growth and sporulation of B. cereus when grown on LAM supplemented with 10 mM L-lysine. The uninhibited enzyme used as a control was assayed as described in the text (●). Inhibited enzyme contained 10 mM D,L-α,ε meso DAP (Δ), 10 mM L-lysine (O), or 10 mM L-lysine plus 10 mM D,L-α,ε meso DAP (▲) in the reaction mixture. The units of specific activity are in umoles/min/mg of protein.

aspartokinase activity altogether. The inhibition curve of aspartokinase by meso DAP is shown in Fig. 10.

DISCUSSION

Aspartokinase activity has been found to be biphasic during growth and sporulation in B. cereus T (Fig. 7, Hoganson and Stahly, 1975b). Evidence is presented here that indicates the two peaks of aspartokinase activity are due to two different isoenzymes of aspartokinase. Figure 8 shows that aspartokinase activity during growth and early stationary phase is sensitive to inhibition by lysine. This aspartokinase activity is called aspartokinase I. As the cells of B. cereus T begin to sporulate the sensitivity of aspartokinase to lysine inhibition is lost; however, DAP added to the reaction mixture is able to inhibit aspartokinase activity. The isoenzyme of aspartokinase that is sensitive to feedback inhibition by DAP is called aspartokinase II.

When B. cereus T is grown on media supplemented with lysine, the early peak of aspartokinase activity is lost, indicating that aspartokinase I is repressed by lysine (Fig. 7). In the presence of lysine or in its absence, aspartokinase activity can be seen to be derepressed as sporulation begins to occur. This activity was primarily sensitive to inhibition by DAP but was not repressed by DAP. Fig. 9 shows that aspartokinase activity

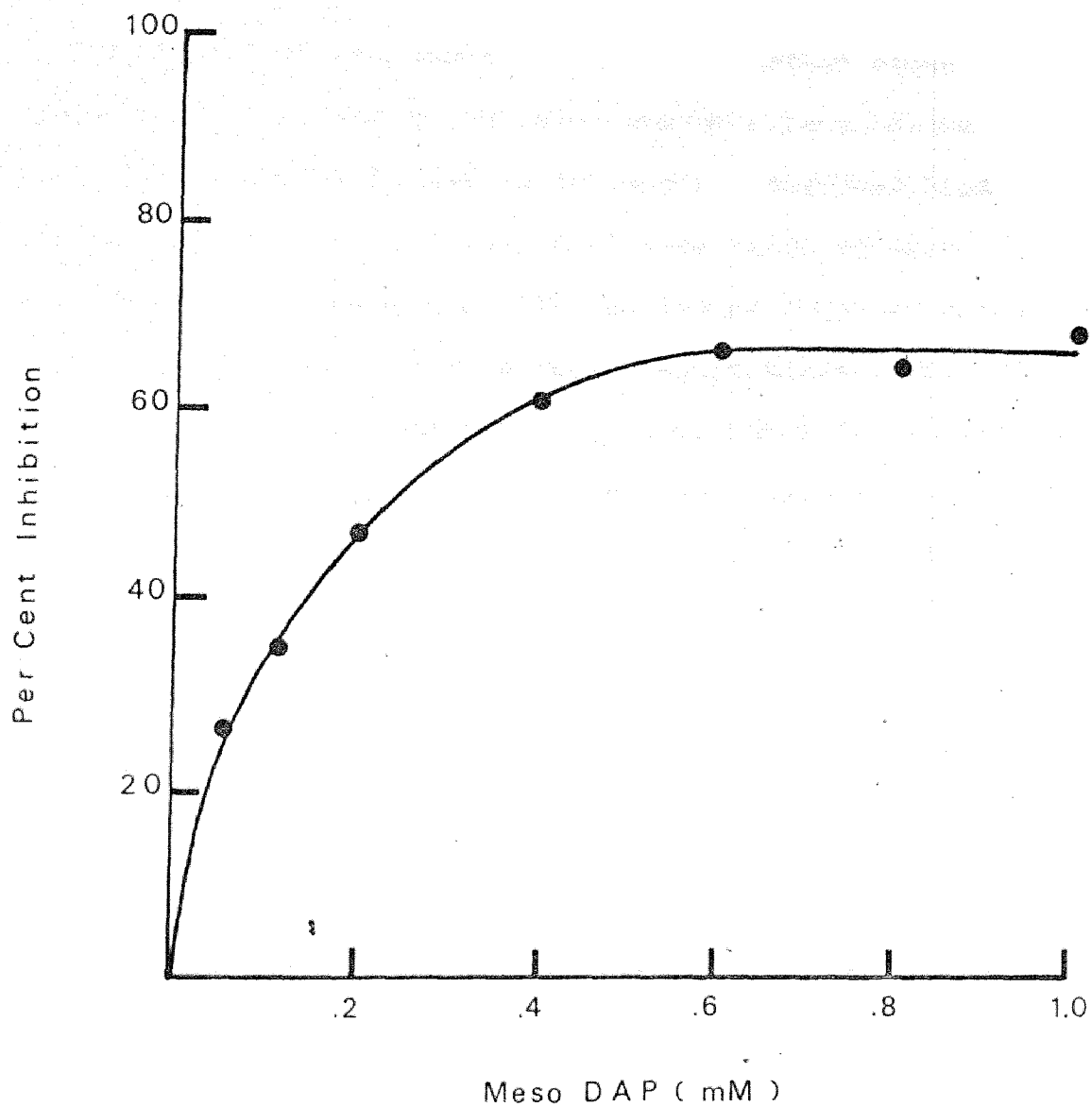

Fig. 10. The effect of meso DAP concentration on the activity of aspartokinase from sporulating cells of Bacillus cereus. The reaction mixture contained 5 mM L-lysine to inhibit the lysine-sensitive aspartokinase in the cell-free extracts.

not repressed by lysine is highly sensitive to inhibition by DAP.

Lysine plus DAP together in the reaction mixture inhibited aspartokinase activity roughly equal to the sum of lysine inhibition alone plus DAP inhibition alone. Therefore inhibition by DAP plus lysine acting in concert on a single aspartokinase is ruled out. Residual aspartokinase activity is seen when cell-free extracts were assayed in the presence of DAP and lysine together which raises the possibility of a third aspartokinase (Figs. 8 and 9). This seems unlikely and is probably due to alterations of the effector site on the enzyme without alteration of the catalytic site during preparation of the cell-free extracts. The results show that while aspartokinase I is primarily found during vegetative growth and aspartokinase II is found primarily during sporulation either isoenzyme may be found at any time during growth and sporulation. This may be of physiological significance to the cell or may be a parameter involved by the batch method of synchronously growing cells.

Aronson et al. (1967) had noted that aspartokinase became insensitive to lysine inhibition during sporulation, but prior to the commencement of DPA synthesis. They ruled out the possibility of two aspartokinases in B. cereus T because mutant #21, a lysine auxotroph with a reversion rate of a point mutation, had a low aspartokinase activity

and produced spores with low amounts of DPA. That is, a single mutation that altered aspartokinase activity impaired the synthesis of lysine which occurs prior to sporulation and DPA which is specific for sporulation. These researchers did acknowledge that an alteration in aspartokinase would take place for lysine insensitive aspartokinase activity to occur. Aspartokinases I and II could share a common subunit, A-1, that is synthesized constitutively. The A-1 subunit would have little catalytic activity until bound with a B-1 subunit to form aspartokinase I or the B-2 subunit to form aspartokinase II. The B-1 subunit might bind with the A-1 subunit giving the moiety catalytic activity and may contain the binding site for the inhibitor. The synthesis of the B-1 subunit would be repressed by lysine along with diaminopimelate decarboxylase synthesis suggesting that these two enzymes may be on the same operon. Similarly the B-2 subunit could form a complex with the A-1 subunit to form a complex with aspartokinase activity sensitive to inhibition by DAP. Furthermore, the B-2 subunit may be coregulated on the same operon coding for aspartate semialdehyde dehydrogenase, dihydrodipicolinate synthase, and dihydrocipicolinate reductase, all of which increase in specific activity during sporulation. A mutation to the A-1 subunit would impair the activity of aspartokinase I and aspartokinase II. Mutant #21 of Aronson et al. (1967) would have a defective A-1 subunit while

mutant HS29 of Forman and Aronson (1972) which had normal activity during log growth but was impaired in DPA production and had low aspartokinase activity during sporulation would have a defective B-2 subunit.

The data of Aronson et al. (1967) indicates that aspartokinase activity during sporulation is stabilized by 2-mercaptoethanol while aspartokinase activity from cells in exponential growth was not affected by 2-mercaptoethanol. This may mean that aspartokinase II is stabilized by 2-mercaptoethanol, however, Hoganson and Stahly (1975b) were only able to get aspartokinase I, i.e., lysine sensitive, activity even though 2-mercaptoethanol was present in the buffer. Hoganson and Stahly (1975b) found lysine to completely repress aspartokinase activity during growth and sporulation. These investigators were unable to explain how ^{14}C-asparate could be incorporated at a normal rate into DPA when lysine had repressed aspartokinase levels. They also found that aspartokinase activity from cells grown in the absence of lysine was inhibited by lysine during exponential growth and sporulation. Apparently, aspartokinase II was being inactivated during preparation of the cell-free extracts despite the presence of 2-mercaptoethanol in the tris-HCl buffer. The results obtained here show higher levels of aspartokinase activity during growth than the results of Hoganson and Stahly (1975b) or any other researchers (Aronson et al., 1967; Forman and Aronson,

1972). Figure 7 shows aspartokinase specific activity insensitive to repression by 10 mM L-lysine which was not found by Hoganson and Stahly (1975b). The higher levels of aspartokinase I and the finding of aspartokinase II may be due to the stabilizing effects of 4 M NaCl in the breakage buffer. It is not known why Hoganson and Stahly were unable to detect aspartokinase II activity while Aronson et al. did.

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